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PATENT SPECIFICATION

NO DRAWINGS

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COMPLETE SPECIFICATION

Diagnostic Composition and method

We, MILES LABORATORIES INC., a Corporation organized and existing under the laws of the State of Indiana, United States of America of 1127, Myrtle Street, Elkhart, Indiana, United States of America, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a urea test composition, and in one of its aspects it relates to a test device useful for the qualitative detection and quantitative determination of urea in fluids, especially but not exclusively body fluids such as blood. In another aspect, it relates to a method of detecting urea in a fluid, other than a body fluid, employing the test device.

In practice, the concentration of urea in blood, for example, is normally expressed in terms of blood urea nitrogen (BUN). This BUN value represents the amount of nitrogen present as urea and is approximately one-half of the total urea value. When either the urea or nitrogen value has been determined, the other value can be calculated therefrom.

The normal range of BUN values in individuals varies between 5—20 mg.%. No significance is ordinarily attached to the lower values. However, elevations in BUN values generally indicate the presence of some abnormal condition. The most common cause of increased blood urea nitrogen is inadequate excretion, usually due to a kidney disease or urinary obstruction. For example, in acute nephritis the BUN level may vary from 25 mg.% to as high as 160 mg.%. Elevated urea retention also occurs with extensive parenchymatous destruction of kidney tissue, as in pyelonephritis, advanced nephrosclerosis, renal tuberculosis, renal cortical necrosis, renal malignancy, renal suppuration or chronic gout. Although BUN values may rise to as high as 400 mg.%, they usually do not exceed 200 mg.%.

A means for the accurate determination of urea in body fluids is of great importance not only in the detection of the above physiological disorders, but also in their control. An individual with a known kidney dysfunction must control his diet or otherwise regulate his protein metabolism and must frequently be guided in this regard by a regular check on the concentration of his blood urea. But beyond its usefulness in regular testing in cases of known kidney dysfunction by both patients and physicians, a urea indicator can also be used efficiently in routine urea analyses of body fluids in hospitals and physicians' offices.

A means for the determination of blood urea nitrogen is of greatest value if such test is conveniently rapid, reliable, simple enough for the technician to learn with ease, accurate enough to serve the clinician and sensitive enough to reflect variations in the patient's condition. Moreover, any composition used in such determinations must be adequately stable.

Procedures for the determinations of urea in various body fluids are well known in clinical chemistry. One such procedure utilizes chemical hydrolysis and requires special apparatus not always available in a routine laboratory. Another procedure employs a direct colorimetric reaction of urea in a protein-free filtrate with an organic reagent such as diacetyl monoxime. Still another involves a test which depends on the action of the enzyme urease to convert urea to an ammonium salt which is measured by titration or nesslerization. These prior procedures have the disadvantage that they all require a considerable amount of skill and familiarity with complicated laboratory techniques. They also require the use of substantial quantities of testing fluid which is not always conveniently available.

[Price 4s. 6d.]

Still more recently (see our Specification No. 922,665) a convenient enzymatic type diagnostic composition for the determination of urea in body fluids has been developed which has greatly simplified BUN determinations. This composition comprises essentially buffered urease and an indicator. However, even in the light of this most important advancement, a need for a more sensitive and more accurate means for determining urea still exists.

This invention provides more sensitive and more accurate means for determining urea, in body and other fluids, including a diagnostic composition for determining urea in fluids which is stable in dry form, and simple, rapid, and extremely sensitive in use, and free of many of the disadvantages of prior compositions.

The new composition comprises urease, preferably water-soluble, a buffering compound capable of producing ammonium ions, an indicator system capable of undergoing a detectable change when the urease acts upon urea present in a fluid, and optionally colour stabilisers and other adjuvants.

The basic enzymic reaction underlying this invention is well known. Urease catalyses the hydrolysis of urea to produce principally ammonia and carbon dioxide. Ammonium carbonate and possibly intermediate compounds such as the unstable carbamic acid may also be formed. This enzymic hydrolysis of urea can be represented by the following reaction sequence:

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease}} [NH_2COOH] \rightarrow CO_2 + NH_3 \rightarrow (NH_4)_2CO_3 + NH_3$$

A product of this hydrolysis can be determined by means of the indicator system. For example, when the indicator system includes a pH indicator comprising a dye that is color sensitive to changes in pH, contact of said system with a product of this hydrolysis, produces a color change in said dye. This color change can be represented by the following equation:

$$\text{pH Indicator (Color A) Acidic} \xrightarrow[\text{obtained from enzymic hydrolysis of urea}]{NH_3 \text{ or } (NH_4)_2CO_3} \text{pH Indicator (Color B) Basic}$$

Urease can be isolated quite readily from numerous bacteria and fungi as well as from various meals, such as jack bean meal. Since there are numerous methods and techniques for isolating urease, the urease which is commercially available possesses a wide range of physical properties. For use in this invention, urease which is water-soluble is preferred. For unexplainable reasons, the water-soluble urease enhances color development and reduces the incubation time which is required for hydrolyzing urea. Incubation time can be defined as that time which urease must remain in contact with urea to effect substantially complete hydrolysis of urea. When a water-soluble urease is used in the composition of this invention, the incubation times are reduced by as much as 50% over incubation times which are required with other types of urease.

In order to ensure maximum urea sensitivity while at the same time effecting the desired degree of pH control, a buffer capable of producing ammonium ions is used in the composition of this invention. If the buffer utilized is incapable of producing ammonium ions, the composition will possess poor urea sensitivity. For example, a diagnostic composition which contains a conventional buffer, such as sodium citrate, in addition to urease, and a pH indicator such as bromthymol blue, is capable of detecting BUN changes in increments of about 50 mg.%. In other words there must be a BUN increase or decrease of about 50 mg.% before the composition is capable of detecting a change. However, when a buffer capable of producing ammonium ions is used in accordance with this invention, incremental BUN changes of 10 mg.% and less can be detected. This is an improvement in sensitivity of about 500% or more.

In addition to improving sensitivity, the ammonium ion producing buffer is also utilized to maintain the composition at its optimum pH. Such optimum pH is a pH which is between the midpoint of the transition interval of the indicator and a point adjacent to this transition interval, but on the acid side thereof. The transition interval is defined as that pH range over which an indicator will exhibit a color change. For example, for bromthymol blue the transition

interval lies between pH 6.0 and 7.6. Therefore, the midpoint of the transition interval of bromthymol blue is 6.8. In this illustration a pH adjacent to the transition interval of bromthymol blue, but on the acid side thereof would be a pH of slightly below 6.0.

Although there are a variety of compounds capable of producing ammonium ions and which may be used in this invention, ammonium salts of weak acids are particularly well suited and are preferred. Examples of such preferred buffers include ammonium citrate, ammonium lactate, ammonium oxalate, ammonium benzoate, ammonium acetate, ammonium salicylate, ammonium stearate, ammonium propionate, ammonium butyrate and ammonium phosphate as well as mixtures of two or more of such buffers.

In addition to using a particular type of buffering compound, it has been found that the sensitivity of the composition of this invention can be further improved by the addition of a sensitizing agent such as an aliphatic amide having a melting point of below about 150° C. Preferred sensitizing agents include saturated and unsaturated aliphatic amides containing from about 2 to about 4 carbon atoms. Examples of such sensitizing agents include acetamide, propionamide, acrylamide, and butyramide.

The concentration of sensitizing agent used can be varied over a relatively wide range. Generally a concentration of about 0.5% to 10% and higher based on the total weight of the composition can be used without denaturing the urease. However, a concentration of sensitizing agent such as acetamide of about 1.5% to 4.0% is preferred.

The compositions of this invention can be incorporated into various forms of test devices. In a preferred embodiment, bibulous strips are impregnated with a composition of this invention. In use, the resulting test device is contacted with a fluid to be tested and the product of the enzymic reaction, if any, is determined colorimetrically. When the impregnated bibulous strip is contacted with a drop of blood, for example, the urease catalyzes the hydrolysis of the urea present in the blood with the resulting formation of ammonia, carbon dioxide and possibly other reaction products. The quantity of urea hydrolyzed will cause a certain increment of pH increase which, in turn, causes a color change in the indicator. A correlation between color change and urea concentration can be made to provide a highly sensitive quantitative determination of urea. For example, when bromthymol blue is used in the composition of this invention, the various shades of color between yellow and blue (the colors within the transition interval of bromthymol blue) can be correlated to correspond to different urea levels, thereby giving a clear visible index of the concentration of urea present in the blood.

Although the ingredients comprising the composition of this invention are preferably impregnated into a bibulous carrier, this invention can also be used as a liquid system. A convenient liquid system can be prepared by lyophilizing a solution of the composition and then reconstituting with the fluid to be tested. If desired, this invention can also be used in the form of tablets, pellets, or powders.

Although the preferred pH indicator is bromthymol blue, other pH indicators can also be used. Such indicators include, for example, bromcresol purple, dichlorosulphonphthalein, 6,8 - dinitro - 2,4 - quinazolinedione, alizarin, and 2 - (2,4 - dinitrophenylazo) - 1 - naphthol 3,6 - disulfonic acid.

In certain instances it is preferable that the color developed when the test composition of the present invention is contacted with urea be such that it does not fade for an interval of 60 seconds or more after development. This can be accomplished by introducing into the composition a color stabilizer. Suitable color stabilizers which can be used include albumins such as bovine albumin and egg albumin. Other protein-type material found in fluids of plants and animals which possess albuminoid properties can also be used. The concentration of albumin added will generally be less than 10% and greater than 0.1% based on the total weight of the composition. However, an albumin concentration of between 1% and 5% by weight is preferred.

If a more stabilized color is desired or needed a dual stabilizing agent may be employed. This dual color stabilizing agent is comprised of two essential ingredients. The first of these two essential ingredients is an albumin. Although normally materials such as bovine albumin and egg albumin are the preferred type of protein material, other protein material, such as that found in fluids of plants and animals and which possess albuminoid properties, can also be used.

The other ingredient is a heteropolysaccharide material. Such materials include, for example, gum arabic (Acacia senegal), mesquite gum (Prosopsis juliflora), Damson gum, gum tragacanth (Astragalus gummifer) and Indian gum (Anogeissus latifolia). The above gums are generally obtained from plants in the form of a thick, mucilaginous water-soluble excretion.

It has been found that optimal color stabilization of the diagnostic composition is obtained when certain weight ratios and particular concentrations of these ingredients are utilized. Preferably the gum material and albumin are combined in a weight ratio of about 2:1. In other words, for every 2 g.

of gum material present in a color stabilizing agent, there is present 1 g. of albumin. However, a weight ratio of gum to albumin as high as 4:1 and as low as 0.5:1 can be used.

The concentration of the dual color stabilizing agent incorporated in a composition should also be controlled within certain limits. Preferably, the color stabilizing agent is present in the composition of this invention in a concentration of about 2% based on the total weight of the composition. However a concentration as high as 4% and as low as 1% can be used.

If desired, various additives may also be incorporated in to the composition of this invention as protective, thickening or wetting agents. For example, thickening agents such as gelatin or wetting agents such as polyvinyl alcohols and solid polyethylene glycols which have a molecular weight of between about 6000 to 7500 can be used. A polyethylene glycol such as "Carbowax 4000" (Registered Trade Mark) which is obtainable from Union Carbide Chemicals Co. is particularly useful. Protective agents in the form of polymeric films can also be used to enhance the quality of the diagnostic compositions of this invention when used with certain forms of test devices, for example, such as bibulous strips. Inert dyes to impart a uniform color background may also be used.

A polymeric film such as a film of ethyl cellulose can be used as a dialyzing membrane to keep the larger molecules present in the fluid being tested, such as haemoglobin in blood, out of contact with the diagnostic composition while at the same time permitting the remaining portions of the fluid, including any urea present, to pass therethrough and contact the test composition. As a result, staining of the test composition by haemoglobin and the masking red color resulting therefrom are avoided. The aforementioned large molecules can be readily washed or wiped off of the polymeric film to permit observation of any resulting color change in the indicator. Further, the polymeric film protects the diagnostic composition from decomposition. Although ethyl cellulose is the preferred polymer, other polymeric materials can be used if desired.

The invention is illustrated in greater detail, but not limited, by the following Examples:

EXAMPLE 1

The composition of this example was formulated as follows:

Gelatin	0.5 g.
Urease	0.5 g.
Acetamide	1.0 g.
4% "Carbowax 4000"	11.5 ml.
0.1M Ammonium Citrate Buffer (dibasic)	2.5 ml.
1.6% aqueous solution Bromthymol Blue	3.8 ml.

The gelatin was added to 11.5 ml. of water and heated until completely dissolved. The remaining ingredients were combined and then mixed with the solution of gelatin until a clear solution was obtained. The temperature of the final solution was approximately 30° C. The composition was then adjusted to a pH of about 6.5 by the addition of small quantities of dilute sodium hydroxide. Paper strips measuring 2 inches by 1/4 inch were then dipped in the adjusted solution and air dried at a temperature of 85° C. The dried strips were then coated with a polymeric film by dipping into a 1.25% solution of ethyl cellulose in benzene, after which they were allowed to air dry until the benzene completely evaporated.

A second composition was prepared which was similar to the above with the exception that the ammonium citrate buffer was replaced with a sodium citrate buffer.

The following testing procedure was then followed in determining BUN levels with both compositions.

The coated ends of both strips were moistened with one drop samples of blood containing known concentrations of urea. After a two-minute incubation period at room temperature the blood was removed by washing with water. The developed colors of both series of strips were immediately compared to color standards which had been previously correlated to indicate the concentration of blood urea nitrogen present in the blood sample. The results obtained are tabulated in Table 1.

TABLE 1

Run	Known Conc. BUN mg. %	Color Chart Readings in Conc. of BUN, mg. %	
		Ammonium Citrate Buffer	Sodium Citrate Buffer
1	10.0	10	20
2	22.1	20	40
3	23.8	25	50
4	34.4	30	75
5	35.4	35	60
6	75.6	75	100
7	81.0	80	100
8	84.8	80	95
9	107.2	100	100
10	162.0	100+	100+

It can be seen that the results obtained with the composition containing sodium citrate as the buffer were erratic. On the other hand, the composition containing the ammonium citrate buffer was extremely sensitive particularly in the lower ranges and coincided very closely to the predetermined BUN concentrations.

EXAMPLE 2

The procedure of Example 1 was repeated with the exception that the acetamide used as sensitizing agent in Example 1 was replaced with acrylamide. The results obtained were comparable to those obtained in Example 1 and accurately reflected the BUN concentration in the blood samples.

EXAMPLE 3

The procedure of Example 1 was repeated except that the ammonium citrate buffer was replaced with ammonium acetate. The results obtained were comparable to those obtained with an ammonium citrate buffer. In all instances, the amount of deviation from the actual urea nitrogen concentration was less than 10 percent.

EXAMPLE 4

The composition of this example was formulated as follows:

Gelatin	0.5 g.
Urease	0.5 g.
Acetamide	1.0 g.
4% "Carbowax 4000"	11.5 ml.
0.1M Ammonium Citrate Buffer (dibasic)	2.5 ml.
1.6% aqueous solution Bromthymol Blue	3.8 ml.
Bovine Albumin	0.5 g.

The gelatin was added to 11.5 ml. of water and heated until completely dissolved. The remaining ingredients, were combined and then mixed with the solution of gelatin until a clear solution was obtained. The temperature of the final solution was approximately 30° C. The composition was then ad-

justed to a pH of about 6.5 by the addition of small quantities of dilute sodium hydroxide. Paper strips measuring 2 inches by 1/4 inch were then dipped in the adjusted solution and air dried at a temperature of 85° C. The dried strips were then coated with a polymeric film by dipping the strips into a 1.25% solution of ethyl cellulose in benzene, after which they were allowed to air dry until the benzene completely evaporated.

A second composition was prepared which was identical to the first composition with the exception that no bovine albumin was added. The following testing procedure was then followed in determining the BUN level with both compositions.

The coated ends of both strips were moistened with one drop samples of blood containing 28 mg.% urea. After a two-minute incubation period at room temperature the blood was removed by washing with water. The developed colors of both series of strips were immediately compared to color standards which had been previously correlated to indicate the concentration of blood urea nitrogen present in the blood sample. After 60 seconds had elapsed, the developed colors of both strips were again compared to color standards.

The composition containing albumin and the composition without albumin indicated that a urea concentration of between 25 mg.% and 30 mg.% was present in the blood being tested. Sixty seconds after the first reading, the colors or hues of both compositions were again interpreted. In this instance, the composition containing albumin indicated a urea concentration of between 20 mg.% and 25 mg.% while the composition without albumin indicated a urea concentration of between 5 mg.% and 10 mg.%. Color interpretations at time intervals of about 30 seconds and 90 seconds gave approximately the same degree of improved accuracy.

EXAMPLE 5

The procedure of Example 4 was repeated with the exception that the sensitizing agent acetamide was replaced with acrylamide and the bovine albumin was replaced with egg albumin. The results obtained were comparable to those obtained in Example 4.

It is apparent from the above example, that the composition containing albumin retained its analytical effectiveness over extended periods of time. The compositions without albumin, on the other hand exhibited analytical inaccuracies if the color interpretation was delayed.

EXAMPLE 6

The composition of this example was formulated as follows:

Gelatin	0.5 g.
Urease (water-soluble)* (225.6 SU units)**	0.04 g.
Acetamide	0.5 g.
4% "Carbowax 4000"	6.0 ml.
0.1M Ammonium Citrate Buffer (dibasic)	1.8 ml.
1.6% aqueous solution Bromthymol Blue	3.8 ml.
Bovine Albumin	0.2 g.
Gum Arabic	0.4 g.

* Water-soluble urease is a highly purified enzyme separated from jack bean and may be obtained through Worthington Biochemical Corp., Freehold, New Jersey.

** A SU (Sumner Unit) of urease activity is that weight in milligrams, which will form 1 milligram of ammonia nitrogen from urea in phosphate buffer, pH 7.0 at 20° C. in 5 minutes.

The gelatin was added to 11.5 ml. of water and heated until completely dissolved. The remaining ingredients, including the albumin and gum arabic, were combined and then mixed with the solution of gelatin until a clear solution was obtained. The temperature of the final solution was approximately 30° C. The composition was then adjusted to a pH of about 6.5 by the addition of small quantities of dilute sodium hydroxide. Paper strips measuring 2 inches by 1/4 inch were then dipped in the adjusted solution and air dried at a temperature of 85° C. The dried strips were then coated with a polymeric film by dipping the strips into a 1.25% solution of ethyl cellulose in benzene, after which they were allowed to air dry until the benzene completely evaporated.

A second composition was prepared exactly like the first composition with the exception that the color stabilizing ingredients, gum arabic and albumin, were removed and a partially water-soluble urease was substituted for the water-soluble urease. The following testing procedure was then followed in determining the BUN levels with both compositions.

The coated ends of both strips were moistened with one drop samples of blood containing known concentrations of urea. After a one-minute incubation time at room temperatures, the blood was removed by washing

with water. The developed colors of both sticks were immediately compared to color standards which had been previously correlated to indicate the concentration of blood urea nitrogen present in the blood sample. After 60 seconds the sticks were again compared with the color standards. This procedure was repeated a number of times with blood samples containing different concentrations of urea. The results obtained are shown in Table 2.

TABLE 2

Run	Known Conc. BUN mg. %	Colour Chart Readings in Conc. of BUN, mg. %			
		Reading Time — Zero Seconds		Reading Time — 60 Seconds	
		Composition with colour stabilizer	Composition without colour stabilizer	Composition with colour stabilizer	Composition without colour stabilizer
11	13	10	10	5	0
12	19	20	20	10	0
13	30	30	20	25	0
14	54	50	50	30	10
15	61	75	75	50	10+
16	105	100	75	75	30

The results obtained, clearly show the improvement which can be obtained when a colour stabilizer comprising gum arabic and albumin is incorporated into a composition containing a water-soluble urease. In all instances there was little colour fading occurring even after a delayed reading time of 60 seconds.

It should be understood that although this invention is of primary interest in determining blood urea nitrogen, it can also be used to test various other body fluids such as urine, serum and saliva and other fluids, known in the art, which may contain urea.

WHAT WE CLAIM IS:—

1. A composition for detecting the presence of urea in a fluid, which comprises urease, a buffering compound capable of producing ammonium ions and an indicator system capable of undergoing a detectable change when the urease acts upon urea present in a fluid.

2. A composition according to claim 1, which further includes a colour stabiliser.

3. A composition according to claim 2, in which the urease is a water-soluble urease.

4. A composition according to any one of the preceding claims which further includes as sensitising agent an amide having a melting point below substantially 150° C.

5. A composition according to claim 1, which further includes as sensitising agent an aliphatic amide having a melting point below substantially 150° C.

6. A composition according to claim 2, which further includes a sensitising agent an aliphatic amide having a melting point below substantially 150° C.

7. A composition according to claim 3, which further includes as sensitising agent an aliphatic amide having a melting point below substantially 150° C.

8. A composition according to claim 2 or 6, in which the stabiliser is an albumin.

9. A composition according to claim 3 or 7 in which the stabiliser is an albumin.

10. A composition according to claim 4 in which the stabiliser is an albumin.

11. A composition according to claim 3 or 7, in which the stabiliser is an albumin and a heteropolysaccharide.

12. A composition according to claim 4, in which the stabiliser is an albumin and a heteropolysaccharide.

13. A composition according to claim 1, substantially as described.

14. A composition according to claim 2, substantially as described.

15. A composition according to claim 3, substantially as described.

16. A composition according to claim 4, substantially as described.

17. A test device which comprises a bibulous carrier impregnated with a composition according to any one of claims 1, 5 and 13.

18. A test device which comprises a bibulous carrier impregnated with a composi-

tion according to any one of claims 2, 6, 8 and 14.

19. A test device which comprises a bibulous carrier impregnated with a composition according to any one of claims 3, 7, 9, 11 and 15.

20. A test device which comprises a bibulous carrier impregnated with a composition according to any one of claims 4, 10, 12 and 16.

21. A test device according to claim 17, coated with a transparent, semipermeable polymeric film.

22. A test device according to claim 18, coated with a transparent, semipermeable polymeric film.

23. A test device according to claim 19, coated with a transparent, semipermeable polymeric film.

24. A test device according to claim 20, coated with a transparent semipermeable polymeric film.

25. A test device according to claim 17 or 21, substantially as described in any one of Examples 1 to 3.

26. A test device according to claim 19 or 23, substantially as described in Example 6.

27. A test device according to claim 20 or 24, substantially as described in any one of the foregoing Examples.

28. A method for detecting urea in a fluid other than a body fluid comprising contacting the fluid with a composition according to any one of claims 1, 5 and 13 or a device according to any one of claims 17, 21 or 25.

29. A method for detecting urea in a fluid other than a body fluid comprising contacting the fluid with a composition according to any one of claims 2, 6, 8 and 14 or a device according to claim 18 or 22.

30. A method for detecting urea in a fluid other than a body fluid comprising contacting the fluid with a composition according to any one of claims 3, 7, 9, 11 and 15 or a device according to any one of claims 19, 23 and 26.

31. A method for detecting urea in a fluid other than a body fluid comprising contacting the fluid with a composition according to any one of claims 4, 10, 12 and 16 or a device according to any one of claims 20, 24 and 27.

32. A method according to claim 28, substantially as described.

33. A method according to claim 29, substantially as described.

34. A method according to claim 30, substantially as described.

35. A method according to claim 31, substantially as described.

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